



Meditation & yoga: Impact on oxidative DNA damage & dysregulated sperm transcripts in male partners of couples with recurrent pregnancy loss

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Background & objectives: Recurrent pregnancy loss (RPL) is one of the devastating complications of pregnancy and current focus lies in addressing the management of paternal factors. Dysregulation in selective transcripts delivered to oocyte at fertilization can result in pregnancy losses and adversely affect embryogenesis. The objective of this study was to assess the effect of yoga-based lifestyle intervention (YBLI) on seminal oxidative stress (OS), DNA damage and spermatozoal transcript levels.

Methods: The present study was a part of a prospective ongoing exploratory study and 30 male partners of couples with RPL were included from August 2016 to June 2017. Semen samples were obtained at baseline and at the end of YBLI (21 days). Gene expression analysis was performed by quantitative polymerase chain reaction on spermatozoal *FOXG1*, *SOX3*, *OGGI*, *PARP1*, *RPS6*, *RBM9*, *RPS17* and *RPL29*. The levels of seminal OS and sperm DNA damage was assessed by measuring levels of reactive oxygen species (ROS) by chemiluminescence and DNA fragmentation index (DFI) by sperm chromatin structure assay.

Results: *SOX3*, *OGGI* and *PARP1* were observed to be upregulated, while *FOXG1*, *RPS6*, *RBM9*, *RPS17* and *RPL29* showed downregulation. A significant reduction in ROS levels, an increase in sperm motility, sperm count (done twice) and a decrease in DFI was seen after YBLI.

Interpretation & conclusions: Adopting YBLI may help in a significant decline in oxidative DNA damage and normalization of sperm transcript levels. This may not only improve pregnancy outcomes but also improve the health trajectory of the offspring.

Key words Dysregulation - meditation - oxidative stress - sperm DNA damage - sperm transcript - yoga

Recurrent pregnancy loss (RPL) is defined as the occurrence of three consecutive miscarriages before 20 completed weeks of gestation¹. With a quantum of accepted aetiopathologies for RPL, the current focus has shifted towards analyzing the paternal

contributions in early embryonic development. Oxidative stress (OS) has been found to be one of the main reasons for defects in sperm function and disrupts the sperm genomic integrity. The vulnerability of the sperm DNA to OS arises due to being transcriptionally

inert, minimal cytosolic antioxidants, a high content of polyunsaturated fatty acid in the plasma membrane and also its deficiency in the DNA damage detection and repair mechanisms^{2,3}. The repair of this oxidative DNA damage (ODD) is henceforth an imperative step for embryo viability and healthy offspring. Because of the insufficient repair mechanisms, the sperm undergoes remarkable levels of chromatin compaction which not only protects the sperm against the DNA damage but is also marked by a shutdown of nuclear transcription⁴⁻⁷. The transcriptionally silent spermatozoa are capable of production of functionally viable transcripts contributing to the transcriptome of the embryo before activation of embryonic genome^{7,8}. These transcripts play a vital role in the translation of proteins involved in critical process related to implantation and early embryonic development⁷⁻¹¹. The dysregulation of these transcripts is associated with pregnancy losses¹⁰⁻¹².

Although genetic and epigenetic factors contribute to the biological makeup of the embryo, various lifestyle and environmental factors have emerged as major risk factors. Complementary and alternative medicine (CAM) has evolved as an important stream of health care. Integrative medicine (IM) approaches representing a cusp of CAM and conventional modern medicine have gained significant interest. Yoga cognitive-behavioural practices have emerged as the most popular IM approaches as these comprise various practices of yoga *asanas*, breathing exercises and mindful meditation¹³. The current study is a part of an ongoing prospective exploratory study for the assessment of the expression of genes critical for embryonic development. Based on previous literature citing studies on sperm RNA profile, the current study was designed to assess the expression of the genes that have been postulated to have a role in early embryonic development^{14,15}. The genes selected for the current study were *FOXG1* (forkhead box G1), *SOX3* (SRY-related HMG-box 3), *RPS6* (ribosomal protein S6), *RBM9* (RNA binding motif protein 9), *RPS17* (ribosomal protein S17) and *RPL29* (ribosomal protein L29) and also the genes of the base excision repair (BER) pathway, *i.e.* *OGG1* (8-oxoguanine DNA glycosylase) and *PARP1* (polyADP-ribose polymerase 1). It becomes pertinent to minimize exposure to factors resulting in the high OS as it can alter sperm epigenome and result in dysregulation in levels of sperm transcripts¹⁶. The present study was aimed to highlight the impact of yoga-based lifestyle intervention (YBLI) in optimizing free radical levels, reducing ODD and normalizing the levels of dysregulated sperm transcripts.

Material & Methods

A total of 42 men who volunteered for the study and gave informed written consent, were recruited for this study for assessing the changes in gene expression with brief YBLI in male partners of couples experiencing idiopathic RPL from August 2016 to June 2017. These couples had consulted the Obstetrics and Gynaecology outpatient department at All India Institute of Medical Sciences (AIIMS), New Delhi, India, and were referred to the Laboratory for Molecular Reproduction and Genetics in the Department of Anatomy. After recording the detailed family history, those with a recent history of any febrile episode, infections or any drug intake in the past three months and also deranged ovarian function, biochemical and hormonal profile in the female partners, were excluded. Only cytogenetically normal cases were included in the study. Of the 42 men included, seven declined to take part, five men were excluded from the study due to failure in compliance and the remaining 30 men were finally enrolled in the programme. Semen samples were also taken from 30 healthy fertile controls who had recently fathered a child within the past two years, but they were not enrolled in the YBLI programme. The study was initiated after obtaining ethical clearance (IECPG-325/29-06-2016) from the Institutional Ethics Committee of AIIMS, New Delhi, India.

Yoga-based lifestyle intervention: All participants were provided with a detailed description of the predesigned YBLI programme¹⁷ with necessary changes as per the patients enrolled in the study. The sessions were conducted for an average of two hours per day under the direct supervision of registered, specialized yoga instructor. This integrative health strategy programme included a series of physical postures (*asanas*), breathing exercises, meditation and each session ended with relaxation through *Shavasana*. The typical session of YBLI involved an interactive session with the patients (Table I).

Laboratory procedures: Semen sample was assessed in the laboratory at the start (day 0) and at the end of YBLI sessions (day 21). Semen analysis was performed as per the WHO guidelines¹⁸. The semen parameters analyzed were volume, pH, viscosity, liquefaction time, motility, morphology and sperm count for screening and recruiting the patients. Sperm count and progressive motility were used in the current study. Total RNA was isolated from 1×10^7

Table I. Details of practices done in a session of Yoga-based Lifestyle Intervention (YBLI) programme

Practices do be done	Duration (min)
Session preparation	5
Starting prayer	3
Loosening practices (<i>Sukshma Vyayama</i>)	5
Sun Salutations (<i>Surya Namaskar</i>) with <i>mool bandha</i>	10
Asanas (postures)	
Standing	
<i>Tadasana</i>	
<i>Ardha chakrasana</i>	
<i>Padahasthasana</i>	
<i>Vrikshasana</i>	
Sitting	
<i>Paschimattanasana</i>	
<i>Janu Sirasana</i>	
<i>Badha Kanasana</i>	
<i>Vakrasana</i>	30
Prone	
<i>Bhujangasana</i>	
<i>Salabhasana</i>	
<i>Naukayasana</i>	
<i>Makrasana</i>	
Supine	
<i>Uttanapadasana</i>	
<i>Malasana</i>	
<i>Pavana mukhtasana</i>	
<i>Matsyasana</i>	
Relaxation	
<i>Shavasana</i>	5
Pranayama (breathing exercises)	
<i>Nadishodhana</i>	
<i>Bhramari</i>	
<i>Kapal Bhati</i>	20
<i>Brahmamudra</i>	
<i>Dhayana</i>	5
Nada anusandhana (A-U-M chanting)	5
Closing prayer	7
Interactive session	25
Total	120

spermatozoa by TRIZOL method¹⁹. RNA quantification was done by spectrophotometry using a NanoDrop spectrophotometer (Thermo-Scientific, Wilmington,

USA). A total of 1000 ng of RNA was used for reverse-transcribing into complementary DNA using iScript cDNA synthesis kit (Bio-Rad, California, USA). Quantitative analysis of each gene was performed using Brilliant III Ultra-Fast SYBR Green quantitative polymerase chain reaction Master Mix by CFX96 real-time system (Bio-Rad, California, USA). The relative quantification of target genes was calculated with $2^{-\Delta\Delta C_t}$ method¹⁹ after normalization of the amount of expressed mRNA using two internal housekeeping genes β -actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Each complementary DNA (cDNA) product was tested in duplicate. Reactive oxygen species (ROS) levels were measured by luminol-dependent chemiluminescence with the luminometer (Sirius; Berthold Detection Systems GmbH, Pforzheim, Germany). The ROS values were expressed as relative light units (RLU)/sec/ 10^6 spermatozoa. The extent of DNA damage was expressed as DNA fragmentation index (DFI) which was measured by sperm chromatin structure assay^{19,20}.

Statistical analysis: Data were analyzed using statistical software, Stata 14.0 (StataCorp LLC, Texas, USA). Quantitative data expressed as mean \pm standard deviation and median (min-max) followed normal and skewed distribution, respectively. Paired *t* test and Wilcoxon signed rank test were used to compare continuous variable both at the start of the intervention (day 0) and at the end of active YBLI (day 21).

Results

The mean age of male partners of couples with RPL who participated in the YBLI intervention was 33.4 \pm 4.7 years. After YBLI a significant increase in sperm count ($P<0.01$) and progressive motility ($P<0.001$) was observed as per two readings taken four days apart. There was a significant (69%) reduction in ROS levels ($P<0.001$), while a minimal non-significant decrease in DFI was also observed (Figure, Table II). The cut-off value for ROS and DFI was set as 27.8 RLU/sec/million sperm and 30.7 per cent, respectively. Relative expression of the genes at the end of YBLI (day 21) as compared to baseline (day 0) was seen to normalize towards that of the control values. The spermatozoal *SOX3*, *OGGI* and *PARP1* were seen to be upregulated, whereas *FOXG1*, *RPS6*, *RBM9*, *RPS17* and *RPL29* were downregulated (Table III).

Table II. Impact of lifestyle intervention on quantitative levels of various experimental parameters at baseline (day 0) and the end of active intervention (day 21)

Experimental parameters	Baseline (day 0)	End of active intervention (day 21)
Sperm count (million/ml)	38.9 (0.4-145.5)	43.4 (1.2-149.2)**
Progressive motility (%)	50 (0-65)	55 (5-75)***
ROS (RLU/sec/million sperm)	39.4 (3.5-1223.1)	12.5 (0.27-142)***
DFI (%)	40.2 (26.8-54.9)	39.0 (24.3-49.8)*

Values given as median (minimum-maximum).

$P^* < 0.05$, $** < 0.01$, $*** < 0.001$ compared to baseline.

ROS, reactive oxygen species; RLU, relative light unit;

DFI, DNA fragmentation index

Table III. Relative expression (average ΔCt) of the GOI with respect to β -actin and *GAPDH* in semen samples of patient as well as controls and AFC in the gene expression post-yoga with respect to pre-yoga

GOI	ΔCt pre-yoga	ΔCt post-yoga	ΔCt controls	AFC
<i>FOXG1</i>	4.6 \pm 1.8	4.2 \pm 0.75	3.9 \pm 1.1	0.49
<i>SOX3</i>	7.6 \pm 0.88	6.4 \pm 1.6	2.7 \pm 1.7	1.67
<i>OGG1</i>	5.4 \pm 0.84	4.8 \pm 1.09	3.9 \pm 2.1	1.3
<i>PARP1</i>	5.9 \pm 1.4	5.3 \pm 0.52	3.5 \pm 1.6	1.8
<i>RPS6</i>	3.3 \pm 1.3	3.06 \pm 1.02	1.9 \pm 1.1	-0.41
<i>RBM9</i>	3.3 \pm 0.63	3.4 \pm 0.44	1.0 \pm 2.8	0.05
<i>RPS17</i>	2.56 \pm 0.91	2.21 \pm 0.63	1.5 \pm 1.09	0.74
<i>RPL29</i>	2.2 \pm 1.3	1.19 \pm 1.1	0.17 \pm 1.76	0.76

Values are mean \pm SD (n=30). GOI, genes of interest; AFC, axis fold change; *FOXG1*, forkhead box G1; *SOX3*, SRY-related HMG-box 3; *RPS6*, ribosomal protein S6; *RBM9*, RNA binding motif protein 9; *RPS17*, ribosomal protein S17; *RPL29*, ribosomal protein L29; *OGG1*, 8-oxoguanine DNA glycosylase; *PARP1*, poly (ADP-ribose) polymerase 1; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase

Discussion

The impact of YBLI in the current study was documented by a significant reduction in OS parameters. A reduction in ROS levels also resulted in a significant improvement in sperm progressive motility, the single most important predictor of fertility potential. A significant improvement in sperm count and also a minimal reduction in DFI following a 21 day YBLI were also seen. Previous studies from our

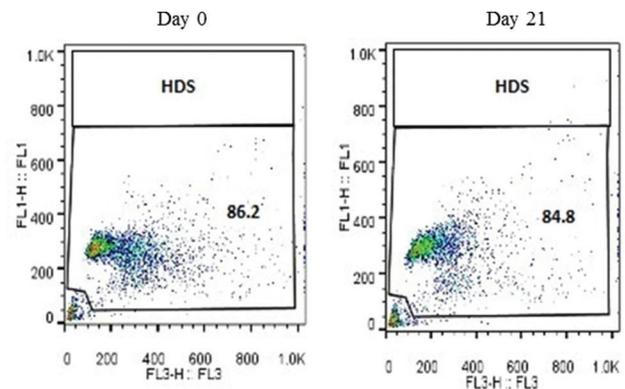


Figure. Dot plot cytograms of semen samples at both baseline (day 0) and at the end of yoga-based lifestyle intervention (day 21) by sperm chromatin structure assay. FL3 on X-axis represents fragmented DNA and FL1 on Y-axis represents native DNA showing percentage of spermatozoa with high levels of DNA fragmentation. HDS represents the percentage of cells with high DNA stainability (%HDS). Each dot in the cytogram represents a single sperm with red and green fluorescence values. The debris at the bottom left corner was excluded from analysis.

laboratory have documented that six months duration of YBLI results in a significant decline in OS, ODD as well as mutagenic load in sperm DNA²⁰⁻²². The patients in the current study were advised to continue the yoga practice at home after the completion of this 21 day YBLI and were followed up to 90-180 days.

OS negatively impacts sperm function by disrupting the sperm DNA integrity as a result of concurrent damage to proteins and lipids present in the plasma membrane. Sperm motility is one of the first functions which is negatively affected by lipid peroxidation and overwhelming OS⁴. Previous studies from our laboratory have documented lower levels of *PARP1* in men with idiopathic infertility²³ which could explain the persistence of DNA damage. The current study showed a minimal non-significant improvement in DFI with 21 days of YBLI and thus helpful for people with ODD to integrate yoga into their lifestyle. Impact of ODD is also witnessed as rapid telomere attrition. Its maintenance is done by telomerase which is an important regulator of telomere length and YBLI exerts a beneficial effect by upregulating telomerase activity and a decline in seminal ROS and ODD^{20,21,24}. Indiscriminate use of antioxidants may cause reductive stress and cause premature nuclear decondensation and impair pronuclear formation, YBLI regulates OS with initial upregulation in antioxidant levels and following their decrease with the practice of YBLI there is downregulation in antioxidant levels^{16,24}.

FOXG1 is a winged helix transcriptional repressor essential for the development of ventral telencephalon and even expressed in neurogenic zones of postnatal brain. It is a key regulator in cortical development in regulating progenitor proliferation and a downregulation of *FOXG1* aids in pyramidal cell migration determining their density in cortex^{25,26}. *SOX3* functions as a transcriptional factor for the formation of the hypothalamo-pituitary axis, male sex determination in the developing foetus, craniofacial morphogenesis and suppresses neuronal differentiation via counteracting proneural protein activity. Both over and underdosage of *SOX3* has been associated with X-linked hypopituitarism and neural tube defects. *SOX3* expression in developing urogenital ridge is responsible for normal functioning of seminiferous tubules and decreased expression suppresses spermatogenesis²⁷. A downregulation of *FOXG1* and upregulation of *SOX3* genes was seen in the patients at the end of YBLI with respect to the baseline in the current study. Furthermore, the relative gene expression (average Δ Ct) was seen to normalize towards that of healthy fertile controls.

Spermatozoal *RPS6*, *RBM9* and *RPL10A* genes positively correlate with higher incidence of miscarriage^{12,15}. *RPS6* is the major substrate of protein kinases in the ribosome. The phosphorylation of *RPS6* has been studied as a marker for neuronal activation, while its dephosphorylation is witnessed at phases of growth arrest²⁸. *RBM9* also known as *RBFOX2* [RNA binding protein, the fox-1 homologue is a member of Rbfox family (*RBFOX1*, *RBFOX2* and *RBFOX3*)], is one of the important regulators of alternative exon splicing in the nervous system and has been shown to be required for embryonic stem cell viability²⁹. A deficiency in any of the Rbfox homologues results in inhibition of late neuronal differentiation in post-meiotic neurons, and one homologue can compensate for the lack of the other²⁹. Both *RPS6* and *RBM9* showed downregulation with respect to the pre-intervention levels and also normalized with respect to the relative gene expression of fertile controls. The impact of YBLI caused a positive change in the expression of genes of the BER pathway. The expression of *PARP1* and *OGG1* involved in DNA damage detection and repair were seen to be upregulated and is an important predictor for the maintenance of genomic integrity. Spermatozoal *RPS17* and *RPL29* were seen to be downregulated in the current study.

As OS impacts the epigenome (by altered methylation levels), it may significantly impact sperm

epigenome. Our earlier study showed upregulation in cellular repair genes and downregulation of pro-apoptotic and pro-inflammatory genes in primary open angle glaucoma patients and a significant decrease in intraocular pressure³⁰. IM practices may alter gene transcription and cause modifications at the level of DNA methylation and histone modifications.

In the present study the changes in gene expression profile in spermatozoa were assessed with simple YBLI and a reduction in seminal OS and improvement in sperm motility in male partners of RPL patients were seen. The current findings need a large sample size for further validation, which is a potential lacuna in the study. The result of this study suggests that integration of yoga/meditation into our daily lifestyle may help to decrease seminal OS, ODD which not only aids in telomere length maintenance but decreases the accumulation of mutagenic bases and normalizes the expression of sperm transcripts and thus positively impacts the dynamic sperm epigenome.

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Conflicts of Interest: None.

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